

CHEMICALLY DEFINED, SYNTHETIC MEDIA FOR SPORULATION AND FOR GERMINATION AND GROWTH OF *BACILLUS SUBTILIS*

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ABSTRACT

DONNELLAN, J. EDWARD, JR. (U.S. Army Natick Laboratories, Natick, Mass.), ELLA H. NAGS, AND HILLEL S. LEVINSON. Chemically defined, synthetic media for sporulation and for germination and growth of *Bacillus subtilis*. J. Bacteriol. 87:332-336. 1964.—From 90 to 130 mg (dry weight) of spores (about 1% dark forms) were obtained (per liter) from a chemically defined, synthetic medium, with a two-phase (polyethylene glycol-potassium phosphate) harvest procedure. Optimal sporulation occurred when glucose and glutamic acid were at a concentration of 10 mM in the medium. Ca^{++} and Mn^{++} were required for sporulation. Heat resistance, dipicolinic acid content, and properties of germination and postgerminative development of spores grown in different concentrations of Ca^{++} were investigated. Heat shock did not increase germination of spores derived from the synthetic medium. A synthetic medium, in which spore germination, emergence, and first cell division approached synchrony, was devised.

For the past 10 years, we used a sporulation medium containing Liver Fraction "B" (Wilson and Co., Chicago, Ill.) buffered with 10 mM phosphate. This medium gives good yields of spores of many species of *Bacillus* (Foster and Heiligman, 1949; Levinson and Sevag, 1953). For example, approximately 350 mg (dry weight) of *B. megaterium* spores, and approximately 250 mg (dry weight) of *B. subtilis* spores are obtained per liter of medium containing 0.5% Liver "B." Indeed, all of the 14 species and 21 strains of *Bacillus* represented in the *Quartermaster Culture Collection* (Reese et al., 1950) sporulate and are maintained on a medium containing nutrient agar supplemented with 0.2% Liver "B." However, we have recently been engaged in experiments aimed at elucidating the kinetics of synthesis of

various biochemical fractions during germination and postgerminative development of bacterial spores, and, for tracer studies, it is advantageous (Roberts et al., 1957) to produce spores on a chemically defined, synthetic medium. For our particular requirements, it was also necessary to devise a synthetic medium on which spores are capable of rapid, synchronous germination and development. In this paper, we describe techniques for producing spores of *B. subtilis*, some effects of varying constituents of the synthetic sporulation medium, and the development of a synthetic medium for germination and postgerminative development.

MATERIALS AND METHODS

Organism. The Marburg strain of *B. subtilis* was received from I. Slotnick of the University of Florida, as American Type Culture Collection strain no. 6051. It corresponded to the species as described by Smith, Gordon, and Clark (1952). Its sporulation characteristics appeared to be similar to those of the strain of *B. subtilis* previously used (Donnellan and Morowitz, 1960).

Sporulation medium. The medium which we devised contained (final concentration) FeCl_2 or FeCl_3 , 0.0036 mM; MgCl_2 , 0.041 mM; MnCl_2 , 0.1 mM; NH_4Cl , 10 mM; Na_2SO_4 , 0.75 mM; KH_2PO_4 , 0.5 mM; CaCl_2 , 1 mM; NH_4NO_3 , 1.2 mM; D-glucose, 10 mM; and L-glutamic acid, 10 mM (pH 7.1). In some respects, it was a modification of the medium suggested by Demain (1953) for germination and growth of various bacilli, and was similar to that used by Krask (1953). Effects of concentration of Ca^{++} , Mn^{++} , glucose, and glutamate on spore yield were examined.

Two methods of preparation and inoculation of the medium proved satisfactory. Salt solutions, without CaCl_2 , were distributed in portions of 125 ml per 1,000-ml Erlenmeyer flask, and were autoclaved. Glucose and L-glutamic acid were dissolved together, neutralized with KOH, and steri-

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lized by Seitz filtration. A water solution of CaCl_2 was autoclaved separately. A water suspension of *B. subtilis*, grown on slants of nutrient agar (Difco) containing 0.2% of Liver Fraction "B," was heat-shocked at 75 C for 15 min, and mixed with the sterile CaCl_2 and glucose-glutamate solutions; 2.0 ml of the mixture were used to inoculate each flask of sterile salt solution. Each flask received 10^6 to 10^7 spores. The second method was similar to the above, except that 115 ml of salt solution, autoclaved in 1,000-ml flasks, were inoculated with 10 ml of the spore- CaCl_2 -glucose-glutamate mixture. Flasks were incubated at 29 C on a reciprocal shaker [95 strokes (3-in.) per min] for 4 days, by which time 60 to 80% of the cells had formed spores free of sporangia. Incubation at 35 C resulted in a greatly reduced yield of spores.

Harvesting procedure. Spores were harvested and separated from vegetative cells and debris by a two-phase polyethylene glycol-potassium phosphate system (Sacks and Alderton, 1961). A 1-liter portion of the medium was centrifuged at 4 C, and the sediment (spores, vegetative cells, and debris) was washed with 500 ml of 50 mM phosphate buffer (pH 5.0). (The phosphate buffer and all other solutions were stored at 4 C prior to use.) The resulting pellet was suspended in 250 ml of distilled water and mixed in a 1-liter beaker with 200 ml of a 1:1 (by weight) aqueous solution of polyethylene glycol-600 (Union Carbide Chemicals Co., New York, N.Y.); 400 ml of 3 M phosphate buffer (pH 7.1) were added. After 3 min in an ice bath, a sharp phase boundary was formed, and the top phase (removed by suction) was mixed in a second 1-liter beaker with 500 ml of 2 M phosphate buffer (pH 7.1). The top phase was again removed, and the spores in this phase were washed seven times with 250 ml of water by centrifugation at 4 C. During the last six washings, the top of the pellet was poured off with the supernatant fluid, as this portion contained a large fraction of germinated spores. If more than 1 liter of spores was harvested at one time, they were combined gradually during the last six water washes, so that the last wash contained all the harvest in one pellet. The resulting pellet was suspended in 15 to 25 ml of distilled water, frozen in a layer on the sides of a 50-ml centrifuge tube, and dried under vacuum for 1 or 2 days. We generally recovered between 90 and 130 mg of spores (about 1% dark forms under phase optics) per liter of medium. In two experiments with poly-

ethylene glycol-4,000 in place of polyethylene glycol-600, 14 and 34 mg of spores per liter of medium were obtained. This sporulation medium was also used successfully for producing spores of *B. megaterium* QM B1551, with yields averaging about 100 mg per liter of medium.

Spore properties. Various properties of spores produced on the synthetic sporulation medium containing various Ca^{++} concentrations were examined. Heat resistance was measured by survival (colony formation on nutrient agar) of water-suspended spores after exposure to 90 C for varying lengths of time. Dipicolinic acid content was determined colorimetrically by the method of Janssen, Lund, and Anderson (1958). For measurement of the capacity of spores produced on the synthetic medium to germinate and grow, aqueous suspensions of spores grown on 10 mM and 1 mM CaCl_2 were heated for 15 min at 55, 70, or 85 C. These heat-shocked spores, or unheated spores, were added to a culture medium in 50-ml Erlenmeyer flasks for a total volume of 3 ml, containing 1 mg of spores per ml, 50 mM glucose, 50 mM KH_2PO_4 , 10 mM L-alanine, 5 mM L-glutamic acid, 5 mM L-asparagine, 1 mM L-lysine, 1 mM DL-methionine, and 0.04 mM MgCl_2 , at pH 7.1. Microscope slide preparations, made at intervals, were scored with a phase contrast microscope for spores (bright); germinated spores (appearing dark under phase optics, but of approximately the same size as spores); emerged cells (longer than spores, generally with ruptured spore coats attached); and dividing cells. This was the medium used in collecting the data presented in Fig. 3.

Media for germination and postgerminative development. Other media were studied for their ability to support rapid and synchronous germination and postgerminative development. These were made by adding a sufficient number of 0.5 or 1.0-ml portions of concentrated salts-amino acids solutions to 50-ml Erlenmeyer flasks to make a final volume of 2.0 or 2.5 ml. The test media were inoculated with 0.5-ml portions of spores suspended in water or phosphate buffer. Amino acid solutions, at five times their final concentration, appeared to interact with glucose. In experiments where such interaction might occur, the glucose was added to the flasks either with the spore inoculum or after inoculation. Most experiments were performed with 1 mg of spores per ml in a total volume of 2.5 to 3.0 ml in 50-ml Erlenmeyer flasks. The same pattern of germination

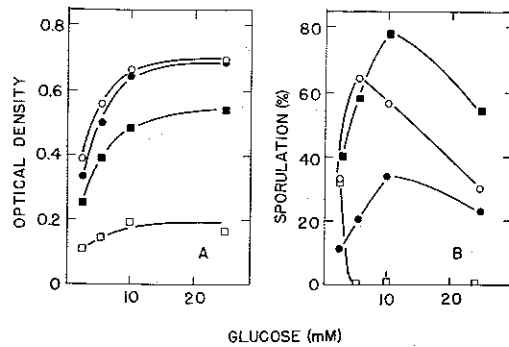


FIG. 1. Effect of concentration of glucose and of L-glutamic acid on growth (A) and sporulation (B) of *Bacillus subtilis*. Incubation (125 ml per 1-liter flask) on reciprocal shaker for 4 days at 29 C. Symbols: □, 2.5 mM; ■, 10 mM; ○, 25 mM; and ●, 50 mM L-glutamic acid.

TABLE 1. Yield of *Bacillus subtilis* spores from synthetic sporulation media containing various concentrations (mM) of Ca^{++} and Mn^{++}

Ca^{++}	Mn^{++}		
	0	0.01	0.1
0	0*	—	<0.1
0.1	0	25	85
0.5	0	84	—

* Results expressed as milligrams of spores harvested per liter of synthetic medium.

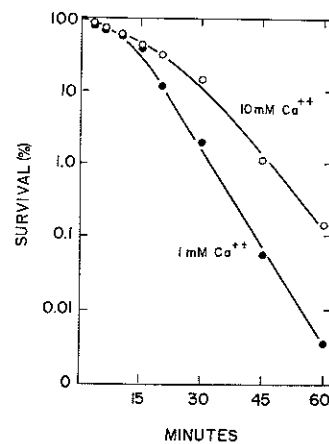


FIG. 2. Effect of Ca^{++} concentration of medium on heat resistance of *Bacillus subtilis* spores. Survival of spores, heated at 90 C for indicated times, was determined by colony formation on nutrient agar (Difco). Each point represents average of at least three batches of spores.

and postgerminative development was obtained with 0.1 mg of spores per ml in a total volume of 100 ml in 1,000-ml Erlenmeyer flasks. Inoculated flasks were incubated on a reciprocal shaker at various temperatures, and germination and postgerminative development were followed with a phase-contrast microscope as described above.

RESULTS AND DISCUSSION

Sporulation medium.

1). *Effect of composition on spore yields.* Glucose was varied from 1 to 25 mM, and L-glutamic acid from 2.5 to 50 mM. The total amount of growth at 4 days increased with increasing concentration of glucose or of glutamate, and leveled off at about 10 mM glucose and at about 25 mM L-glutamic acid (Fig. 1A). On the other hand, the percentage of sporulation reached a maximum, but then de-

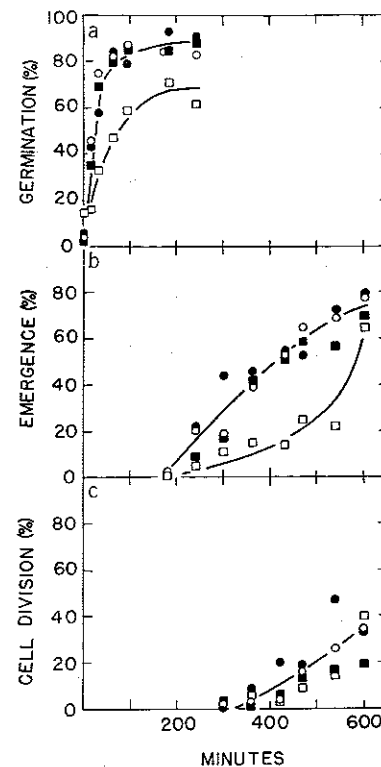


FIG. 3. Effect of temperature of heat shock on *Bacillus subtilis* spore germination, emergence, and cell division. Spores produced on medium containing 1 mM Ca^{++} ; medium for germination and postgerminative development described in Materials and Methods section on spore properties. Aqueous suspensions of unheated (●) spores or of spores heated 15 min at 55 C (■); 70 C (○); or 85 C (□).

creased when the glucose or glutamate concentration exceeded 10 mM (Fig. 1B). Glucose and certain nitrogenous compounds were shown to be ineffective or detrimental to sporulation of *B. cereus* var. *mycoides* (Foster, 1956) and of *B. megaterium* (Grelet, 1957). Ca^{++} and Mn^{++} are required for *B. subtilis* spore production on the synthetic medium (Table 1; see also Charney, Fisher, and Hegarty, 1951; Slepecky and Foster, 1959).

2). *Effect of Ca^{++} on spore properties.* Heat resistance increased with Ca^{++} increase from 1 to 10 mM (Fig. 2), confirming the observations of Sugiyama (1951) and of Slepecky and Foster (1959). Spores (of various batches) contained from 75 to 91 μg of dipicolinic acid per mg of spores; this was independent of concentrations of Ca^{++} between 0.1 and 10 mM in the growth medium.

Heat shock for 15 min at temperatures up to 85 C had no effect on the germination of spores which had been produced on the synthetic sporulation medium containing 10 mM CaCl_2 . However, when the spores were produced in the presence of 1 mM CaCl_2 , the total amount of ger-

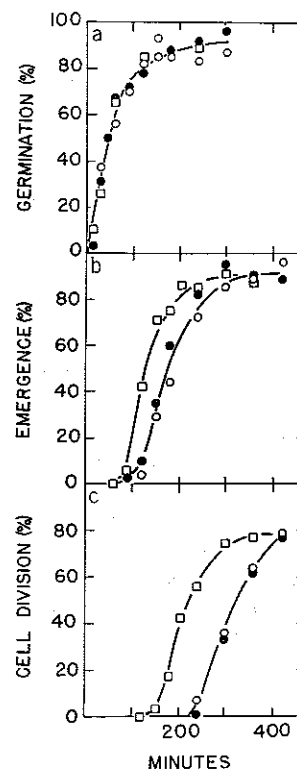


FIG. 4. *Bacillus subtilis* spore germination, emergence, and cell division on synthetic (●) and on Casamino Acids (○) media (Table 2). Initial spore concentration, 1 mg per ml; initial pH, 7.5; incubation, 30 C; □, same as ● but incubated at 35 C.

TABLE 2. Composition of synthetic and of Casamino Acids media for *Bacillus subtilis* spore germination, emergence, and cell division

Ingredient	Concn* in	
	Casamino Acids medium	Synthetic medium
Casamino Acids.....	1	
L-Valine.....	—	20
L-Arginine·HCl.....	—	2.4
L-Leucine.....	—	7.0
L-Threonine.....	—	4.2
L-Serine.....	—	6.0
L-Glutamic acid.....	25	25
L-Alanine.....	14	14
L-Asparagine.....	10	10
D-Glucose.....	50	25
MgCl ₂	0.04	0.4
KH ₂ PO ₄	50	10
Na ₂ SO ₄	0.75	0.75
CaCl ₂	0.1	0.1
FeCl ₂	0.0036	0.0036
NH ₄ Cl.....	10	10
MnCl ₂	0.1	0.1
NH ₄ NO ₃	1.2	1.2

* All concentrations are millimolar, with the exception of Casamino Acids which is a percentage.

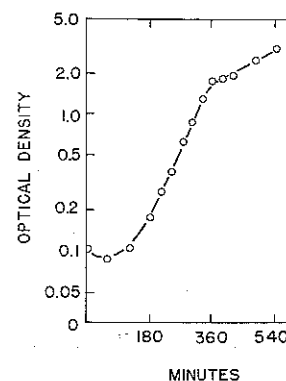


FIG. 5. Growth of *Bacillus subtilis* on synthetic medium for germination and postgerminative development. Optical density (550 mμ) of culture, diluted 1:5, measured with Zeiss model MM 12 spectrophotometer. Inoculated with spores to give an initial concentration of 0.1 mg of spores per ml. Volume, 100 ml in 1,000-ml Erlenmeyer flasks; temperature, 35 C.

mination was decreased by heat shock at 85 C, but not at 70 C (Fig. 3A). Spores grown in either concentration of CaCl_2 , and heated at 85 C, showed an initially decreased rate of emergence, but emergence eventually reached the same level as with spores which had been heated at temperatures up to 70 C (Fig. 3B, for spores grown in 1 mM Ca^{++}). Heat shock did not appear to affect cell division of spores derived from media containing either concentration of Ca^{++} (Fig. 3C).

Synthetic medium for germination and postgerminative development. The medium for spore germination and postgerminative development used above (Fig. 3) was not particularly suitable for our purposes, both because of the relatively small amount of cell division and because of the asynchronous character of emergence. A similar medium, but with added salts and Casamino Acids (Table 2), gave greatly improved results (Fig. 4). By substituting 17 amino acids for Casamino Acids, in the concentrations in which they occur in the Casamino Acids medium, essentially similar results were obtained. With this "synthetic" Casamino Acids medium as a starting point, factorial-type experiments aimed at elimination of harmful or unnecessary amino acids were performed. Those amino acids or combinations of amino acids giving a picture of germination, emergence, and cell division similar to that obtained with the Casamino Acids medium were included in the defined germination and postgerminative development medium finally selected. The final synthetic medium is given in Table 2, and the results of a typical experiment are shown in Fig. 4. The near-synchronous germination and postgerminative development is apparent when these results are compared with those of Fig. 3. Postgerminative development on this defined medium was more rapid at 35 C than at 30 C, but the rate of germination was not increased by this increase in incubation temperature (Fig. 4). Figure 5 is a typical growth curve obtained with this medium. The lag phase lasted approximately 120 min, and the exponential phase, about 190 min, under these conditions. The early decrease in optical density coincided with spore germination (Powell, 1950).

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